



The
Patent
Office

PCT/GB 99/01450

INVESTOR IN PEOPLE

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

REC'D 15 JUN 1999
WIPO PCT

GB99/1450

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

An Brewster

Dated 4 June 1999

THIS PAGE BLANK (USPTO)

11 MAY 1998

13MAY98 E359776-1 D02000
P01/7700 25.00 - 9810085.2 The Patent OfficeCardiff Road
Newport
Gwent NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an information leaflet from the Patent Office to help you fill in this form.)

1. Your reference GWS/DJC/20535

2. Patent application number
(The Patent Office will fill in this part)

9810085.2

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Gemini Research Ltd.
Eagle House
110 Jermyn Street
LONDON SW1Y 6RH

(a) Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

7474640001



4. Title of the invention POLYMORPHISM IN A NITRIC OXIDE SYNTHASE GENE

5. Name of your agent (if you have one)

MATHYS & SQUIRE
100 GRAY'S INN ROAD
LONDON WC1X 8AL

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

(b) Patents ADP number (if you know it)

1021001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country Priority application number (if you know it) Date of filing (day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application Date of filing (day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

9. Enter the number of sheets for any of the following items you are filing with this form.
Do not count copies of the same document

Continuation sheets of this form

Description	17
-------------	----

Claim(s)	2
----------	---

Abstract	1
----------	---

Drawing(s)	2 + 2
------------	-------

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination
(Patents Form 10/77)

Any other documents
(please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature Mathy & Squire Date
MATHYS & SQUIRE

11 MAY 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

GEORGE W. SCHLICH

TEL: 0171 830 0000

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- b) Write your answers in capital letters using black ink or you may type them.
- c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- e) Once you have filled in the form you must remember to sign and date it.
- f) For details of the fee and ways to pay please contact the Patent Office.

Polymorphism in a nitric oxide synthase gene

This invention relates to a polymorphism in a nitric oxide synthase (NOS) gene and to diagnostic method and apparatus based upon the polymorphism, in particular a polymorphism that is located within the promoter region of the gene. The invention also relates to methods of identifying individuals having a predisposition or susceptibility to essential hypertension and also to the group of conditions that contribute to Syndrome X and to methods of treating those individuals to prevent, delay or reduce disease.

Essential hypertension is a common multifactorial disorder that affects approximately 20% of Caucasian adults. It results in a significantly increased risk for heart attack and stroke. The condition has a genetic basis, although at present the number of genes is unknown. Hypertension is also known to cluster with obesity and other disorders such as non-insulin dependent diabetes (NIDDM), atherosclerosis, vascular disease and dyslipidaemia in a metabolic syndrome known as Syndrome X. If the genes that cause susceptibility to Syndrome X disorders can be identified, then treatments for specific gene defects can be targeted.

Inducible NOS (iNOS) is expressed in a variety of tissues including myocytes, small vessel endothelium, vascular smooth muscle cells, hepatocytes, renal proximal tubule, Henle's loop, macula densa, afferent arteriol, astrocytes and immune cells¹⁷. Nitric oxide, produced by either constitutive or inducible isoforms of NOS, influences smooth muscle vasodilation suggesting that it may play a role in regulating blood pressure¹⁸. However, no genetic link between NOS genotype and hypertension has so far been identified.

Three members of the iNOS II gene family NOS2A, NOS2B and NOS2C have been co-localized to human chromosome 17 between bands p13.1 and q25⁵. Furthermore, the human NOS2A gene, localised to 17cen - q11.2¹⁴, contains a biallelic 4 bp repeat polymorphism located within the promoter¹³.

At present, the only available treatments for hypertensive disorders are pharmaceutical based medications that are not targeted to an individual's actual defect; examples include ACE inhibitors and diuretics for hypertension, insulin supplementation for NIDDM, cholesterol reduction strategies for dyslipidaemia, anti-coagulants, β blockers for cardiovascular disorders and weight reduction strategies for obesity. If targeted treatment strategies were available it might be possible to predict the response to a particular regime of therapy and could markedly increase the effectiveness of such treatment. Although targeted therapy requires accurate diagnostic tests for disease susceptibility, once these tests are developed the opportunity to utilise targeted therapy will become widespread. Such diagnostic tests could initially serve to identify individuals at most risk of hypertension and could allow them to make changes in lifestyle or diet that would serve as preventative measures. The benefits associated by coupling the diagnostic tests with a system of targeted therapy could include the reduction in dosage of administered drugs and thus the amount of unpleasant side effects suffered by an individual. In more severe cases a diagnostic test may suggest that earlier surgical intervention would be useful in preventing a further deterioration in condition.

It is an object of the invention to provide genetic diagnosis of predisposition or susceptibility to Syndrome X, and to hypertension in particular. Another related object is to provide treatment to reduce or prevent or delay the onset of disease in those predisposed or susceptible to this disease. A further object is to provide means for carrying out this diagnosis.

Accordingly, a first aspect of the invention provides a method of diagnosis of disease in an individual, said method comprising determining the genotype of a NOS gene in said individual.

In another aspect, the invention provides a method of identifying an individual predisposed or susceptible to a disease, said method comprising determining the genotype of a NOS gene in said individual.

The invention is of advantage in that it enables diagnosis of a disease or of certain disease states via genetic analysis which can yield useable results before onset of disease symptoms, or before onset of severe symptoms. The invention is further of advantage in that it enables diagnosis of predisposition or susceptibility to a disease or of certain disease states via genetic analysis.

The invention may also be of use in confirming or corroborating the results of other diagnostic methods. The diagnosis of the invention may thus suitably be used either as an isolated technique or in combination with other methods and apparatus for diagnosis, in which latter case the invention provides a further test on which a diagnosis may be assessed.

The present invention stems from using allelic association as a method for genotyping individuals; allowing the investigation of the molecular genetic basis of essential hypertension. In a specific embodiment the invention tests for the presence of a 4 base pair insertion in a repeat sequence within the promoter of the NOS2A gene. The invention demonstrates a link between this tetranucleotide insertion and predisposition to essential hypertension by showing that an increased number of hypertensives when compared to normotensives possess the NOS2A 4 base pair promoter insertion.

Certain disease states would benefit, that is to say the suffering of the patient may be reduced or prevented or delayed, by administration of treatment or therapy in advance of disease appearance; this can be more reliably carried out if advance diagnosis of predisposition or susceptibility to disease can be diagnosed.

In a particular embodiment of the invention, the method comprises determining genotype of a repeat region located 5' to the coding sequence within the promoter of an iNOS gene. The polymorphism is preferably a four base pair insertion located in the region -891 to -575 base pairs 5' to the transcription start site. The present invention in a specific example describes the location of a hypertension susceptibility locus on chromosome 17 and specifically implicates the inducible

nitric oxide synthase gene, NOS2A, at 17cen-q11.2. Accordingly the invention provides strong evidence for a chromosome 17 role in human essential hypertension.

The method of the invention optionally comprises determining whether an individual is homozygous or heterozygous for polymorphisms of said iNOS gene. A determination that an individual is free of a risk genotype may provide a more significant diagnosis. Likewise, presence of two risk alleles may give a significant diagnosis of predisposition to disease, and particularly hypertension.

In an embodiment of the invention, the disease is Syndrome X. The invention thus assists in identifying those individuals predisposed or susceptible to this syndrome, enabling early commencement of therapy or treatment or other techniques to avoid or reduce the disease, these latter including adopting a different lifestyle or a different diet. A number of individual disorders are known to be contained within or typically contribute to or feature in Syndrome X and references to Syndrome X are intended to be references to one or more diseases selected from the group consisting of hypertension, obesity, non-insulin dependent diabetes, atherosclerosis, dyslipidaemia, vascular and coronary artery disease.

It is therefore a further aspect of the invention to provide a method of treatment of an individual comprising determining genotype of a promoter region of an iNOS gene, determining if that individual is predisposed or susceptible to Syndrome X and if that individual is so diagnosed providing treatment to reduce or delay or prevent disease.

Current treatments and therapies for Syndrome X are all of application in the present invention for treatment and therapy for an individual diagnosed as predisposed or susceptible to Syndrome X. Insulin supplements are suitable for non-insulin dependent diabetes. A strategy to reduce cholesterol intake is suitable for dyslipidaemia. Anti-coagulants and β -blockers are suitable for cardiovascular disorders. Weight reduction strategy is suitable for obesity.

In a specific embodiment of the invention there is provided diagnosis of predisposition or susceptibility to hypertension. Suitable hypertension treatments are disclosed in US-A-5510390, 5496569, 5405872 and 5409936, the contents of which are incorporated herein by reference. Accordingly, the method may further comprise a treatment selected from the group consisting of administration of an effective amount of antihypertensive pharmaceutical, administration of an effective anti-hypertension therapy or administration of both an effective anti-hypertension therapy and an effective amount of antihypertensive pharmaceutical.

Anti-hypertension therapy may include correction of obesity, high alcohol intake, high salt intake and/or lack of regular exercise. Anti-hypertensive pharmaceuticals may include beta-adrenoceptor blocking drugs, optionally in combination with a thiazide, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors, vasodilators, alpha-blockers and centrally acting drugs such as prazosin, terazosin and doxazosin. One embodiment of the present invention is that a particular polymorphism may indicate that a certain course of treatment could meet with a greater level of success than other treatments. Thus an advantage of the present invention is that it could assist in the choice of which of the many available therapies should be administered to the patient.

Determination of the genotype of said iNOS gene is suitably accomplished by screening the promoter region of said iNOS gene to identify a polymorphism in said 5' region of said iNOS gene, said polymorphism being indicative of a risk genotype in said individual. In an embodiment of the invention, the screening is accomplished by a technique selected from the group of techniques consisting of amplification of a nucleic acid sequence located in said 5' region of the iNOS gene, Southern Blotting of said 5' region of the iNOS gene and single strand conformational polymorphism (SSCP) mapping of said 5' region of the iNOS gene. The invention also encompasses screening the whole or a part of the promoter region of an iNOS gene for a polymorphism in linkage disequilibrium with a polymorphism in or near the 5' region of the iNOS gene.

The invention further encompasses the identification of other polymorphisms that are correlated with a known polymorphism in or near the 5' promoter region of an iNOS gene consisting of:-

- (a) locating a polymorphism and correlating it with the known NOS gene polymorphism; and
- (b) testing whether the new polymorphism is linked to Syndrome X or any contributory component thereof.

At present there is one other known polymorphism in the NOS2A promoter, located between 2.7 and 2.5 kb upstream of the transcription start site³¹. This polymorphism consists of a CCTTT pentanucleotide repeat and has a heterozygosity of 80.2%. In humans the number of these CCTTT repeats present in the promoter can vary from 9 to 16. Any effect of this particular polymorphism on transcription of the NOS2A gene is yet to be confirmed and it may therefore be linked with a predisposition to essential hypertension.

A specific example of the invention described in more detail below, uses one or more primers which will, following conventional polymerase chain reaction (PCR) techniques, amplify a nucleic acid sequence located in said promoter region of the NOS2A gene in particular between positions -891 and -575 base pairs 5' to the transcription start site. The product of the PCR includes an amplified nucleic acid sequence (SEQ ID NO:1). The next step is to determine the size of the amplified sequence. A suitable method is capillary electrophoresis, in which nucleic acids of different sizes migrate in a medium, typically a gel, at a rate according to their size. Two particular PCR primers (ref. 13) have a nucleotide sequence selected from the group of nucleotide sequences consisting of SEQ ID NO:2 and SEQ ID NO:3, though other primers may be used for this purpose.

SEQ ID NO:2

5' TGGTGCATGCCTGTAGTCC

SEQ ID NO:3

5' GAGGCCTCTGAGATGTTGGTC

These two primers are adapted to amplify a nucleic acid sequence located within said promoter region of the NOS2A gene. A risk genotype incorporates a four base insertion allele of 317 bp in size, a non-risk genotype incorporates a wild type allele of 313 bp in size, and the diagnosis of the invention may be carried out in particular on a human.

The invention also provides use of means to determine genotype of a promoter region of an iNOS gene in manufacture of apparatus for diagnosis of predisposition or susceptibility to Syndrome X. In an embodiment of this aspect of the invention, the PCR primers are adapted to amplify a fragment located within said promoter region of said gene, which region consists of or comprises positions -891 and -575 base pairs 5' to the transcription start site.

The invention still further provides a kit for diagnosis of predisposition or susceptibility to Syndrome X comprising one or more primer nucleic acid molecules for determining genotype of promoter region of an iNOS gene and apparatus for correlating iNOS genotype with risk of predisposition or susceptibility to disease. In a specific embodiment of the invention said apparatus for correlating iNOS genotype with risk comprises a set of reference markers that are run on a gel alongside the products of the PCR reaction. In another specific embodiment of the invention said apparatus comprises a set of reference gels that are compared to the gel on which the PCR products have been run thus allowing determination of a risk genotype. In a further specific embodiment of the invention said apparatus comprises a chart on which is indicated the size of the PCR products that allow identification of a risk genotype.

A preferred kit of the invention comprises PCR primers adapted to distinguish between risk and non-risk genotypes of a promoter region of an iNOS gene. Particularly preferred is one comprising primers adapted for amplification of the

whole or a fragment of a region consisting of or comprising positions -891 and -575 base pairs 5' to the transcription start site, such as primers SEQ ID NO:2 and SEQ ID NO:3.

According to the present invention, there is a significant association of the tested polymorphism marker in the Syndrome X disease hypertension. There now follows a brief description of particular embodiments of the invention which are illustrated by drawings in which:-

Figure 1 shows a physical and genetic map (sex averaged) of chromosome 17 markers used in linkage analysis; chromosomal order and interval distances were obtained from Genethon data (refs. 22,23); the positioning of the iNOS gene (NOS2A) is based on Genome Database (GDB)²⁴ and Genetic Location Database (LDB)²⁵ data; and

Figure 2 shows a region of the human iNOS gene that contains the tetranucleotide insertion and that is suitable for diagnosis according to the present invention.

Example 1:

The NOS2A tetranucleotide repeat polymorphism was tested for linkage in 177 hypertensive sibpairs and for allelic association in 77 hypertensive and 76 normotensive individuals. SPLINK results indicated significant excess allele sharing with the biallelic NOS2A polymorphism ($P = 0.0002$). ASPEX^{15,16}, a recently released analysis package, which uses an alternate restriction to SPLINK when performing maximum likelihood calculations, was also used to analyze NOS2A linkage data. Results using ASPEX indicated significant excess allele sharing and linkage of NOS2A in our hypertensive sibpair population (MLOD = 4.4). In addition, allelic association, as tested by chi-square analysis, indicated a significant association of the NOS2A polymorphism with hypertension ($\chi^2 = 5.9$; $P = 0.016$). As shown in Table 2, an increased number of hypertensives (19%) compared to normotensives (9%) possessed the NOS2A 4 bp promoter insertion. The odds ratio

for hypertension associated with this insertion was estimated to be 2.3 (95% CI = 1.1-4.8).

Subjects. Blood from 239 Caucasian hypertensive siblings (blood pressure \geq 140/90 mmHg prior to anti-hypertensive medication) was collected from contacts obtained through the National Health and Medical Research Council of Australia (NHMRC) Twin Registry and also from general practitioners and media releases. In addition, for the allelic association studies, blood was collected from 77 hypertensives (blood pressure \geq 140/90 mmHg prior to medication and who were the offspring of two hypertensive parents) and from 76 normotensives (blood pressure $<$ 140/90 mmHg and who were the offspring of two normotensive parents), as previously described²⁰. A detailed questionnaire was completed by all participants to obtain demographic parameters, to determine ancestry and to exclude those with a family history of diabetes and thyroid disease.

Genotyping. Genomic DNA was extracted from blood samples and markers genotyped using PCR and capillary electrophoresis, as previously described²¹. Fluorescently labelled primers were used to amplify DNA to detect the NOS2A biallelic marker¹³. All PCR products were genotyped using an ABI PRISM 310 Genetic Analyzer with GeneScan Software (Applied Biosystems, Foster City, CA).

Statistical analysis. Genotypes for the affected sibpairs were assessed and analyzed for linkage using both identity by state (IBS) and identity by descent (IBD) nonparametric methods. The extent of allele sharing was determined using the affected pedigree member (APM)^{9,10}, SPLINK^{8,9} and ASPEX^{15,16} statistical packages. For APM analysis, maximum-likelihood estimates of allele frequencies for the 13 markers used in the chromosome 17 scan, were calculated from hypertensive sibship data, using the USERM13 program²⁵ of the MENDEL package of programs²⁶. For SPLINK and ASPEX analysis, maximum-likelihood estimates of the allele frequencies were internally calculated. Maximization of the likelihood ratio for SPLINK analysis was restricted to the possible triangle restriction (ie. $z[1] < 0.5$ and $z[0] < 0.5 \times z[1]$) (refs. 7,8). Additionally, to allow for a moderate amount

of genetic dominance variance, maximum likelihood calculations for ASPEX (aspxe.phase) analysis of NOS2A linkage data, assumed the following multiplicative model for z values: $z[2] = y^2$, $z[1] = 2y(1-y)$ and $z[0] = (1-y)^2$, where y is the sharing at this locus, derived using a moderate sibling recurrence risk ratio (λ_s) of 1.6 (refs. 15,16). In hypertension, there is some suggestive evidence for modest dominance variance due to higher correlation values for systolic and diastolic blood pressure between pairs of siblings than between pairs of parent and offspring (genetic dominance variance estimated at 0.18 and 0.22 for systolic and diastolic blood pressure, respectively)²⁷. Allelic association results for NOS2A were analyzed by the chi-square test. Odds ratio and 95% confidence limit calculations were performed using the Epi Info Version 6 statistical program²⁸.

Table 1 Relevant characteristics of hypertensive siblings

Category	n	Measurement
Pre-treatment systolic BP (mmHg)	99	169.4 ± 24.0
Pre-treatment diastolic BP (mmHg)	99	102.2 ± 10.2
BMI (kg/m ²)	210	27.2 ± 5.2
Age (years)	227	55 ± 11
Siblings - male	84	
- female	155	
⇒ total	239	
Sibpairs - male : male	23	
- female :	79	
female		
- female : male	75	
⇒ total	177	

Data are mean ± standard deviation; BP = blood pressure; BMI = body mass index

Table 2 Association analysis of *NOS2A* in hypertensive and normotensive subjects

Population	n	Genotypes (bp)			Alleles (bp)	
		313/313	313/317	317/317	313	317
Hypertensives	77	55 (0.71)	15 (0.20)	7 (0.09)	125 (0.81)	29 (0.19)
Normotensives	76	64 (0.84)	10 (0.13)	2 (0.03)	138 (0.91)	14 (0.09)

Chi-square analysis of total allele counts for the *NOS2A* polymorphism indicated a significant difference between the hypertensive and normotensive groups ($\chi^2 = 5.9$; $P = 0.016$, df = 1). The odds ratio for hypertension associated with the 4 bp insertion is 2.3 (317 bp versus 313 bp allele; 95% CI 1.1-4.8).

References

1. Mattei, M.-G. *et al.* Angiotensin-I converting enzyme gene is on chromosome 17. *Cytogenet. Cell Genet.* 51, 1041 (1989).
2. Jeunemaitre, X., Lifton, R.P., Hunt, S.C., Williams, R.R. & Lalouel, J.-M. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. *Nature Genet.* 1, 72-75 (1992).
3. Townsend-Nicholson, A., Baker, E., Sutherland, G.R. & Schofield, P.R. Localization of the adenosine A_{2b} receptor subtype gene (*ADORA2B*) to chromosome 17p11.2-p12 by FISH and PCR screening of somatic cell hybrids. *Genomics* 25, 605-607 (1995).
4. Hoehe, M.R. *et al.* Genetic linkage of the human gene for phenylethanolamine N-methyltransferase (*PNMT*), the adrenaline-synthesizing enzyme, to DNA markers on chromosome 17q21-q22. *Hum. Mol. Genet.* 1, 175-178 (1992).
5. Bloch, K.D. *et al.* Three members of the nitric oxide synthase II gene family (*NOS2A*, *NOS2B*, and *NOS2C*) colocalize to human chromosome 17. *Genomics* 27, 526-530 (1995).
6. Hilbert P, *et al.* Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. *Nature* 353, 521-529 (1991).
7. Holmans, P. Asymptotic properties of affected sib-pair linkage analysis. *Am. J. Hum. Genet.* 52, 362-374 (1993).
8. Holmans, P. & Clayton, D. Efficiency of typing unaffected relatives in an affected-sib-pair linkage study with single-locus and multiple tightly linked markers. *Am. J. Hum. Genet.* 57, 1221-1232 (1995).
9. Weeks, D.E. & Lange, K. The affected-pedigree-member method of linkage analysis. *Am. J. Hum. Genet.* 42, 315-326 (1988).
10. Schroeder, M., Brown, D.L. & Weeks, D.E. Improved programs for the affected-pedigree-member method of linkage analysis. *Genet. Epidemiol.* 11, 67-74 (1994).
11. Julier, C. *et al.* Genetic susceptibility for human familial essential hypertension in a region of homology with blood pressure linkage on rat

- chromosome 10. *Hum. Mol. Genet.* **6**, 2077-2085 (1997).
12. Singh, A., Sventek, P., Lariviere, R., Thibault, G. & Schiffrin, E.L. Inducible nitric oxide synthase in vascular smooth muscle cells from prehypertensive spontaneously hypertensive rats. *Am. J. Hypertens.* **9**, 867-877 (1996).
13. Bellamy, R. & Hill, A.V. A bi-allelic tetranucleotide repeat in the promoter of the human inducible nitric oxide synthase gene. *Clin. Genet.* **52**, 192-193 (1997).
14. Chartrain, N.A. *et al.* Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J. Biol. Chem.* **269**, 6765-6772 (1994).
15. Hauser, E.R., Boehnke, M., Guo, S.W. & Risch, N. Affected-sib-pair interval mapping and exclusion for complex genetic traits: sampling consideration. *Genet. Epidemiol.* **13**, 117-137 (1996).
16. Hinds, D. and Risch, N. The ASPEX package: affected sib-pair mapping. <ftp://lahmed.stanford.edu/pub/aspx> (1996).
17. Hare, J.M. & Colucci, W.S. Role of nitric oxide in the regulation of myocardial function. *Prog. Cardiovasc. Dis.* **38**, 155-166 (1995).
18. Rapport, R.M. & Murad, F. Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. *Circ. Res.* **52**, 352-357 (1983).
19. Deng, A.Y. & Rapp, J.P. Locus for the inducible, but not constitutive, nitric oxide synthase cosegregates with blood pressure in the Dahl salt-sensitive rat. *J. Clin. Invest.* **95**, 2170-2177 (1995).
20. Rutherford, S. *et al.* Association of a low density lipoprotein receptor microsatellite variant with obesity. *Int. J. Obes. Relat. Metab. Disord.* **21**, 1032-1037 (1997).
21. Nyholt, D.R., Lea, R.A., Goadsby, P.J., Brimage, P.J. & Griffiths, L.R. Familial typical migraine: linkage to chromosome 19p13 and evidence for genetic heterogeneity. *Neurology* **50**, (in press).
22. Gyapay, G. *et al.* The 1993-94 Genethon human genetic linkage map. *Nature Genet.* **7**, 246-339 (1994).

23. Colette, D. *et al.* A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* **380**, 152-154 (1996).
24. Johns Hopkins University School of Medicine, host. The Genome Database. Baltimore. (<http://gdbwww.gdb.org/gdb/gdbtop.html>) (1998).
25. Boehnke, M. Allele frequency estimation from data on relatives. *Am. J. Hum. Genet.* **48**, 22-25 (1991).
26. Lange, K., Weeks, D. & Boehnke, M. Programs for pedigree analysis: MENDEL, FISHER, and DGENE. *Genet. Epidemiol.* **5**, 471-472 (1988).
27. Tambs, K. *et al.* Genetic and environmental effects on blood pressure in a Norwegian sample. *Genet. Epidemiol.* **9**, 11-26 (1992).
28. Dean, A.G. *et al.* Epi Info, Version 6: a word processing, database, and statistics program for public health on IBM-compatible microcomputers. Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A. (1996).
29. Collins, A., Frezal, J., Teague, J. & Morton, N.E. A metric map of humans: 23,500 loci in 850 bands. *Proc. Natl. Acad. Sci. USA* **93**, 14771-14775 (1996).
30. Chambers, S.M. & Morris, B.J. Glucagon receptor gene mutation in essential hypertension. *Nature Genet* **12**, 122 (1996)
31. Xu, W., Liu, L., Emson, P.C., Harrington, C.R. & Charles, I.G. Evolution of a homopurine-homopyrimidine pentanucleotide repeat sequence upstream of the human inducible nitric oxide synthase gene. *Gene*, **204**, 165-70 (1997)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: GEMINI INTERNATIONAL HOLDINGS LIMITED
- (B) STREET: LE REGINA, 13-15 BOULEVARD DES MOULINS
- (C) CITY: MONACO
- (E) COUNTRY: MC
- (F) POSTAL CODE (ZIP): 98000

(ii) TITLE OF INVENTION: POLYMORPHISM IN A NITRIC OXIDE
SYNTHASE GENE

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 317 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGGTGCATGC CTGTAGTCCC AGCTACTCA	60
GAGGCTGAGG TGGGAGGGATC GCTTGAGCCT	
GGGAGGCAGA AGTTGCAATG AGCAGAGATC GTGCCACTCC GCTCCAGTCT TGGTGACAGA	120
ATGAGACTCC ATCTCAAAAA TAAATAAATA AATAAAATAA ATAATGAAA TGAAATTATA	180
AGAAATTACC ACTTTTCAT GTAAGAAGTG ATCATTCCA TTATAAGGGA AGGAATTAA	240
TCCTACCTGC CATTCCACCA AAGCTTACCT AGTGTAAAG GATGAGGTGT TAGTAAGACC	300
AACATCTCAG AGGCCTC	317

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGGTGCATGC CTGTAGTCC

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAGGCCTCTG AGATGTTGGT C

21

Claims:

1. A method for diagnosing disease or a predisposition to disease comprising determining genotype of a NOS gene.
2. A method according to claim 1, comprising determining genotype of an inducible nitric oxide synthase (iNOS) gene.
3. A method according to claim 2, comprising determining genotype of a transcriptional control sequence of the iNOS gene.
4. A method according to claim 3, comprising determining genotype of a promoter region of the iNOS gene.
5. A method according to any of claims 1 to 4 comprising determining whether an individual is homozygous or heterozygous for a risk polymorphism in a NOS gene.
6. A method as in claim 5, wherein the risk polymorphism is a four base pair insertion located between positions -891 and -575 5' to the transcription start site in the promoter of the iNOS gene.
7. A method according to any previous claim for diagnosing Syndrome X or predisposition to Syndrome X, or a contributory component thereof.
8. A method according to claim 7 for the diagnosis of hypertension.
9. A method of diagnosis and treatment of hypertension comprising diagnosing hypertension or predisposition thereto according to claim 8, and treating an individual to reduce, prevent or otherwise ameliorate hypertension.
10. A method of predicting response to hypertension therapy, comprising

diagnosing genotype of a NOS gene.

11. A method of diagnosing Syndrome X or predisposition to Syndrome X comprising screening the whole of or a part of an iNOS gene for a polymorphism in linkage disequilibrium with a polymorphism in or near the promoter region of an iNOS gene.

12. A method according to claim 11 for diagnosing hypertension or predisposition to hypertension.

13. A method of locating a further polymorphism correlated with a known polymorphism in or near the promoter region of an iNOS gene comprising:

- (a) locating a further polymorphism and correlating it with the known NOS gene polymorphism; and
- (b) testing whether the further polymorphism is linked to Syndrome X or any contributory component thereof.

14. A kit for diagnosis of predisposition or susceptibility to Syndrome X comprising:-

- (a) one or more PCR primers for determining genotype of an NOS gene; and
- (b) apparatus for correlating NOS genotype with risk of predisposition or susceptibility to disease.

15. A kit according to claim 14, wherein said apparatus comprises a set of reference markers.

16. A kit according to claim 14, wherein said apparatus comprises a reference gel.

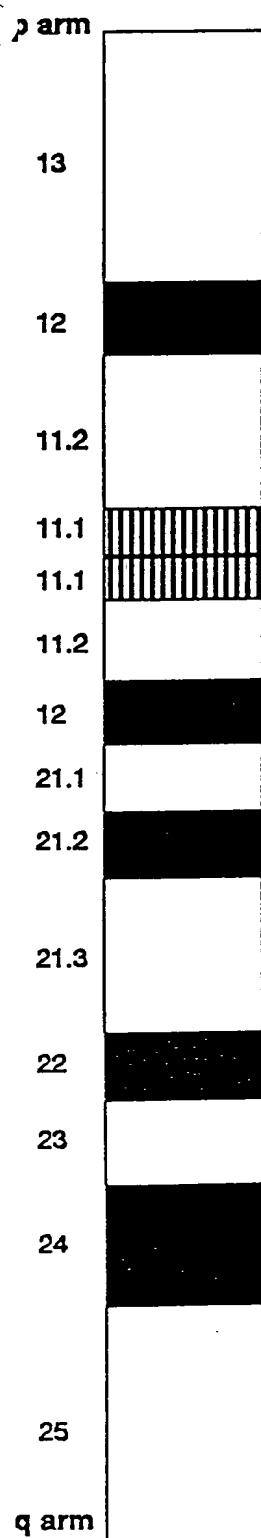
17. A kit according to claim 14, wherein said apparatus comprises a reference chart.

Polymorphism in a nitric oxide synthase Gene

Abstract

A method of diagnosis of disease or predisposition to disease, in particular Syndrome X or a component thereof such as hypertension, comprising genotyping an inducible nitric oxide synthase gene. The presence of a four base insertion polymorphism in the promoter region of said gene denotes an individual with increased risk of disease, in particular hypertension and/or the group of conditions known as Syndrome X.

Chr 17



theta (θ)

D17S849

13

D17S938

7

D17S945

11

D17S799

16

D17S925

6

D17S798

11

D17S791

11

D17S787

8

D17S808

13

D17S949

14

D17S802

10

D17S784

11

D17S928

Fig. 1

NOS2A

THIS PAGE BLANK (USPTO)

fig 2.

TGGTGCATGC CTGTAGTCCC AGCTACTCAG GAGGCTGAGG TGGGAGGATC GCTTGAGCCT	60
GGGAGGCAGA AGTTGCAATG AGCAGAGATC GTGCCACTCC GCTCCAGTCT TGGTGACAGA	120
ATGAGACTCC ATCTAAAAAA TAAATAAATA AATAAATAAA ATAATGAAA TGAAATTATA	180
AGAAAATTACC ACTTTTCAT GTAAGAAGTG ATCATTCCA TTATAAGGGA AGGAATTAA	240
TCCTACCTGC CATTCCACCA AAGCTTACCT AGTGCTAAAG GATGAGGTGT TAGTAAGACC	300
AACATCTCAG AGGCCTC	317

Mathys + Squire
7/15/99

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)